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Evaluation of antimicrobial, antioxidant and wound-healing potentials of *Holoptelea integrifolia*

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Abstract

The methanolic extracts of *Holoptelea integrifolia* (Roxb.) (Urticaceae) leaves (MLE) and stem bark (MSBE) were studied for the wound-healing potential. Since wound healing is severely hampered by microbial infection and reactive oxygen species (ROS), this study was undertaken to evaluate antimicrobial and antioxidant activity apart from wound-healing activity. The antimicrobial property of the *Holoptelea* was studied against the six bacterial and five fungal strains using the agar well diffusion method and minimum microbicidal concentration and minimum inhibitory concentration were determined for each strain, in which methanolic extract of stem bark (MSBE) has shown bigger zone of inhibition (11.3–20.4 mm) than methanolic extract of leaves (MLE) (9.6–14.9 mm). The anti-oxidant activity was evaluated by DPPH free radical scavenging activity using HPLC method. The IC_{50} values obtained for MSBE (TPC: 78.53 ± 1.26 mg/g) and MLE (TPC: 57.71 ± 1.45 mg/g) were 37.66 ± 0.48 and 50.36 ± 0.59 μg/well, respectively. In excision wound model, more than 90% wound healing was recorded in treated groups by 14 days of post surgery, where as only 62.99% was observed in the control group. In incision model, higher breaking strengths and higher hydroxyproline content in treated groups suggested higher collagen re-deposition than the control group. Finally, histopathology studies conformed wound-healing activity of *Holoptelea integrifolia*.

Keywords: *Holoptelea integrifolia* (Roxb.) (Urticaceae); Tensile strength; Minimum inhibition concentration; Mimimum microbicidal concentration; Total phenolic content; DPPH

1. Introduction

Holoptelea integrifolia belongs to the family of Urticaceae. It is an important pollen allergen of India and sensitizes almost 10% of the atopic population in Delhi (Sharma et al., 2005). Recently, an "All India Coordinated Project on Aeroallergens and Human Health" was undertaken to discover the quantitative and qualitative prevalence of aerosols at 18 different centers in the country and found *Holoptelea* is one of the predominant allergen (Singh and Kumar, 2003). Until today, no pharmacological evaluations have been reported on this plant except one group who claimed antiviral activity (Rajbhandari et al., 2001).

Ethnomedically, the leaves and stem bark of this plant were used by local people for skin diseases, obesity (Bambhole and Jiddewar, 1985), cancer (Graham et al., 2000) and for wound healing in the form of paste. The fresh material, either stem bark or leaves of the plant, is applied as paste externally twice or thrice a day for wound-healing purpose.

The process of wound-healing involves inflammation, cell proliferation and contraction of collagen lattice formation (Sidhu et al., 1999). When wound occurs, it is accompanied, with in a short time by pain, reddening and edema, which are the classical symptoms of inflammation. These symptoms are caused by the release of eicosanoids, prostaglandins, leukotrienes and reactive oxygen species (ROS). Since, ROS is produced in high amounts at the site of wound as a defense mechanism against invading bacteria. At the same time, the process of wound healing may be hampered by the presence of free radicals, which can damage wound surrounding cells, or by microbial infection (Houghton et al., 2005). Hence, this study was under taken to evaluate the antioxidant and antimicrobial activity of methanolic extracts of *Holoptelea integrifolia* apart from the wound-healing activity.

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The methanolic extract of leaves (MLE) and stem bark (MSBE) of *Holoptelea integrifolia* were screened for antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (Blois, 1958), and total phenolic content was also estimated. Through the healing process takes place by itself, an infection can seriously delay healing process (Priya et al., 2002). In this context, antimicrobial potentials of MLE (methanolic leaves extract) and MSBE (methanolic stem bark extract) were studied against a wide range of microorganisms. Minimum inhibition concentrations (MICs) and minimum microbicidal concentration (MMC) were determined for each microorganism against the MLE and MSBE. Wound-healing activity was evaluated by taking wound contraction, wound closure, tensile strength and collagen formation as parameters.

2. Materials and methods

2.1. Plant material

Holoptelea integrifolia was collected from Thurkapally, Nalgonda district, of Andhra Pradesh, India in the month of June. The plant was authenticated by Prof. Prabhakar, Department of Botany, Osmania University, Andhra Pradesh, India, and a voucher specimen has been deposited in authors' laboratory under specimen number PVD-01.

2.2. Chemicals and drugs

Methanol and diethyl ether were obtained from Merck Ltd. (Mumbai, India). Gallic acid, ascorbic acid, Folin-Ciocalteu reagent, phenol red, DPPH and Tris were from Sigma Chemicals (St. Louis, MO, USA), nutrient agar was from Himedia (Mumbai, India). Hard paraffin was from Qualigens (India) and cetostearyl alcohol, wool fat and Na_2Co_3 were from Sd fine (India).

2.3. Microorganisms and animals

Wister rats weighing 160–180 g were obtained from National Institute of Nutrition (CPCSEA Reg. No. 154, dated 22 October 1999), Hyderabad registered by Government of India. The animals were housed in a standard individual metal cages and room maintained at $22 \pm 1\,^{\circ}C$ with an alternating 12 h light–dark cycle. Food and water were provided *ad libitum*. All the experiments on animals were conducted after obtaining permission form Institutional Animal Ethical Committee of the institute. Bacterial (*Bacillus subtilis*, *Bacillus cerculences*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella aeruginosa*) and fungal cultures (*Candida albicans*, *Saccharomyces serviseae*, *Aspergillus niger*, *Candida Tropicana* and *Candida krusei*) were procured from MTCC (Microbial Type Culture Collection) Chandhigarh, India.

2.4. Extraction procedure, fingerprinting and phytochemical analysis

Around 300 g of fresh plant material (leaves and stem bark) was washed with tap water, air dried and then chopped into small fragments which are shade dried and reduced to coarse powder with mortar and pestle. The powdered material was extracted three times with hexane to (2.5 l), remove wax, and then extracted three times with methanol (2.5 l), at room temperature in cycle of 48 h each on orbital shaker. The combined methanolic extracts were then concentrated in rotavapour (Ratavac, Heidolf, Germany) at reduced pressure below 40 °C.

Extracts were formulated in to 2% simple ointment (BP) by mixing wool fat (5%), hard paraffin (5%), cetostearyl alcohol (5%), white soft paraffin (83%) and extracts (2%). These ingredients were mixed and heated with gentle stirring until homogenous ointment is formed.

HPTLC fingerprinting: chromatography was performed on 5 cm × 10 cm HPTLC plates coated with 0.25 mm layer of silica gel $60F_{254}$ (Merck, Darmstadt, Germany). Before using, the plates were washed with methanol and activated at 110 °C for 5 min. Samples were applied as 4 mm wide bands and 6 mm apart by using a Camag (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 100 μl syringe. A constant application rate of 6 μl/s was used. Mobile phase for MLE was hexane:ethyl acetate (2:1) and for MSBE was chloroform:methanol:water (5:2:0.5) and chromatograms were monitored at 280 nm.

Phytochemical analysis: the presence of phytochemicals, alkaloids (Draggendorff's), flavonoids (Shibata's reaction), saponins (Frothing test), tannins (5% ferric chloride), terpenoids(2,4-dinitrophenylhydrazine), glycosides (fehling's solution), steroids (Liebermann's Burchard test) and anthraquinones (Borntrager's test), were evaluated according to the methods described by Edeoga1 et al. (2005).

2.5. Pharmacological evaluation

2.5.1. Antioxidant activity

2.5.1.1. Total phenolic content estimation (TPC). Total phenolic content (TPC) was analyzed by the Folin-Ciocalteu colorimetric method using gallic acid as a standard (Yu et al., 2003). Each reaction mixture contained 100 μl of standard gallic acid solution or 100 μl sample solution, 6 ml distilled-deionized water, 500 μl FCR and 1.5 ml of Na_2CO_3 (20 g/100 ml). The reagent blank was performed by replacing the gallic acid solution with 100 μl of acetone–water (1:1, v/v). After 2 h of reaction at ambient temperature, the absorbance of each reaction mixture was measured at 765 nm (SPECTRAmax PLUS®, Molecular Devices, USA). TPC was expressed as mg gallic acid equivalents (GAE) per gram of dry mass of extracts.

2.5.1.2. DPPH free radical scavenging activity by HPLC method. The DPPH free radical scavenging activity was evaluated by the method described by Chandrasekar et al. (2006), with minor modification. Briefly, the reaction mixer consisted of 125 μl of phosphate buffer (pH 7.4, 0.1 mM), 100 μl of DPPH (4.9 mg/25 ml) and 25 μl of test compound and the reaction mixer was kept at room temperature for 5 min, then the sample is filtered through 0.2 μm Nylon membrane filter (Pall Gelman Laboratory, USA) and an aliquot (50 μl) of the sample is injected for HPLC analysis. The blank was prepared by adding 25 μl of

methanol to DPPH and buffer mixer. The HPLC system consisted of a Shimadzu HPLC system (LC-10 Ai, Japan) consisting of pump (LC-10 Ai), a system controller (SCL-10AVP), an auto-injector (SIL-10 ADVP) and a diode array detector (SPD-M10 AVP). Data analysis and processing were done by class LC10 software (Version 1.6). Analysis was done using Kromasil 100 C18 column (250 mm × 4 mm, 5 μM) (Southborough, MA). Isocratic elution was carried out with methanol–water (80:20, v/v) at a flow rate of 1.2 ml/min and the DPPH peaks were monitored at 517 nm. The difference in the reduction of DPPH peak area (A) between the blank and the sample was used for determining the percent radical scavenging activity of the sample. Percent of inhibition was calculated using the following formula:

$$\text{radical scavenging}\ (\%) = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

2.5.2. Antimicrobial activity

2.5.2.1. Sensitivity test (agar diffusion method). Sensitivity test was performed on *Bacillus subtilis*, *Bacillus cerculences*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella aeruginosa*, *Candida albicans*, *Saccharomyces serviseae*, *Aspergillus niger*, *Candida tropicana*, *Candida krusei* according to the method described by Berghe and Vlietinck (1991). Briefly, 30 mg of crude methanolic extracts of either the leaves or bark of *Holoptelea* was dissolved in 1 ml of HPLC grade methanol and filtered through 0.2 μm Nylon membrane filter (Pall Gelman Laboratory, USA) for further use. Culture media were prepared using 40 g/l nutrient agar and were autoclaved at 121 °C, 15 psi for 15 min. A volume of 20 ml of agar was dispersed on petridish and allowed to solidify. Each petridish is divided into four sectors, and in each sector a 4 mm bore was made using borer. Each bore in different sector was loaded with 50 μl of test compound followed by 10 μl of inoculam and incubated for 24 h at 37 °C for bacteria and 28 °C for fungi. Results of the quantitative screening were recorded as the average diameter of the inhibition zone (IZD) surrounding the wells containing the test solution. Chloramphenicol and Amphotericin B were used as standards for bacteria and fungus, at a concentration of 30 μg/μl, respectively.

2.5.2.2. Minimal Inhibitory concentration determination (MIC). Minimum inhibition concentration assay was performed in nutrient broth containing 0.05% phenol red and supplemented with 10% glucose (NBGP), method described by Zgoda and Porter (2001). All the test compounds including standard drugs were initially dissolved in DMSO and the solution obtained was added to NBGP to a final concentration of 5000 μg/ml for each crude extract. This was serially diluted by twofold, to obtain concentration ranging from 5000 μg to 1.22 μg/ml. One hundred microlitres of each concentration was added to a well (96-well microplate) containing 95 μl of NBGP and 5 μl of standard inoculum, the appropriate inoculum size for standard MIC is 2×10^4 to 10^5 CFU/ml. The final concentration of DMSO in the well was less than 1%. The negative control well consisted of 195 μl of NBGP and 5 μl of the standard inoculum. The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37 °C for 24 h (Innova Incubator, Newbrunswick Scientific, USA). The assay was repeated twice and microbial growth was determined by observing the change of colour in the wells (red when there is no growth and yellow when there is growth). The lowest concentration showing no colour change in the well was considered as the MIC.

2.5.2.3. Minimum microbicidal concentrations (MMC). For the determination of MMC, a portion of liquid (5 μl) from each well that showed no change in colour was plated on agar plate and incubated at 37 °C for 24 h. The lowest concentrations that yielded no growth after this sub-culture were taken as the MMC.

2.5.3. Wound-healing models

2.5.3.1. Excision wound model. For excision wound study, male Wister rats (160–180 g) were selected and were divided into four groups of six animals each. Rats were anesthetized with anesthetic ether and depilated at the predetermined site before wounding. An excision wound was inflicted by cutting away approximately 500 mm^2 full thickness of the predetermined area on the anterior-dorsal side of each rat (Dash et al., 2001). Each rat was kept in a separate polypropylene cage and was provided with food and water *ad libitum*.

Control group (Group I) received test compound free simple ointment. In one of the experiment group (Group II), the leaf paste (5%, w/w) applied externally at a final concentration of 50 mg per wound area, another experiment group (Group III) received stem bark paste at a concentration of 50 mg per 500 mm^2 wound area and the positive control group (Group IV) received reference standard Nitrofurazone (NFZ), 0.2% (w/w). All the test formulations were applied once a day for 19 days starting from the day of wounding. Wound-healing property was evaluated by wound contraction percentage and wound closure time. The wound area was measured immediately by placing a transparent paper over the wound and tracing it out, area of this impression was calculated using the graph sheet (Werner et al., 1994). The same procedure is employed every third day and wound contraction was expressed as percentage of contraction. Wound closure time was recorded when total wound healed.

2.5.3.2. Incision wound model. Animals were divided into four groups, Group I control, Group II standard and Group III and Group IV are experimental groups (six animals each) for MLE and MSBE, respectively. All animals of four groups were anesthetized with anesthetic ether, and a paravertebral long incision of 4 cm length were made through the skin and cutaneous muscle at a distance about 1.5 cm from the middle on right side of the depilated back. After the incision was made, the two ends of the wound were drawn closer and sutured at 0.5 cm intervals using sterile surgical thread (No. 000) and a curved needle (No. 11) (Udupa et al., 1995). All the groups were treated as the same in the excision model. Ointments, standard were applied once daily for 9 days. On day 9, sutures were removed and the tensile strength of healed wounds was measured on day10 by Universal Testing Machine (Schimadzu, Switzerland) (Saha et al., 1997).

Tensile strength was calculated using the following formula:

$$\text{tensile strength} = \frac{\text{breaking strength (g)}}{\text{cross sectional area of skin (mm}^2\text{)}}$$

2.5.3.3. Hydroxyproline estimation. On the day 21 of the post surgery of excision, a piece of skin from the healed wound area was collected and analyzed for hydroxyproline content, which is a basic constituent of collagen. Tissues were dried in a hot air oven at 60–70 °C to constant weight and were hydrolysed in 6N HCl at 130 °C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and colour was developed with the help of Ehrlich reagent at 60 °C (Woessner, 1961) and measured at 557 nm using UV/vis spectrophotometer (SPECTRAmax PLUS®, Molecular Devices, USA).

2.5.3.4. Histopathological studies. Skin specimens from treated and untreated rats were collected in 10% buffered formalin and after the usual processing, 5 μm-thick sections were cut and stained with haematoxylin and eosin (McManus and Mowry, 1965). Sections were qualitatively assessed under the light microscope and graded with respect to fibroblast proliferation, collagen formation, epithelisation, keratinisation and scar formation.

2.6. Statistical analysis

All treated groups were compared with the control group and the results were analyzed statistically using one-way ANOVA and followed by Dennett test to identify the differences between treated groups and control. The data were considered significant at $p < 0.05$.

3. Results

3.1. Extraction yield, fingerprinting and phytochemical estimation

Fresh 300 g of leaves and stem bark of *Holoptelea integrifolia* yielded 10.63 (3.54%) and 5.26 g (1.42%) of hexane extracts and 42.03 and 57.23 g of methanolic extracts, respectively.

In the HPTLC fingerprinting of MLE gave 12 spots at the following Rf values: 0.02, 0.12, 0.27, 0.37, 0.43, 0.58, 0.69, 0.76, 0.86, 0.91, 0.95 and 0.97 whereas MSBE gave 13 spots at the following Rf values: 0.05, 0.11, 0.20, 0.26, 0.38, 0.43, 0.53, 0.61, 0.68, 0.73, 0.81, 0.87 and 0.95. The corresponding HPTLC chromatograms are presented in Fig. 1.

The phytochemical analysis of MLE has shown the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides and absence of steroids and anthraquinones. The phytochemical analysis of MSBE has shown presence of alkaloids, flavonoids, tannins, terpenoids and the absence of saponins, steroids and anthraquinones.

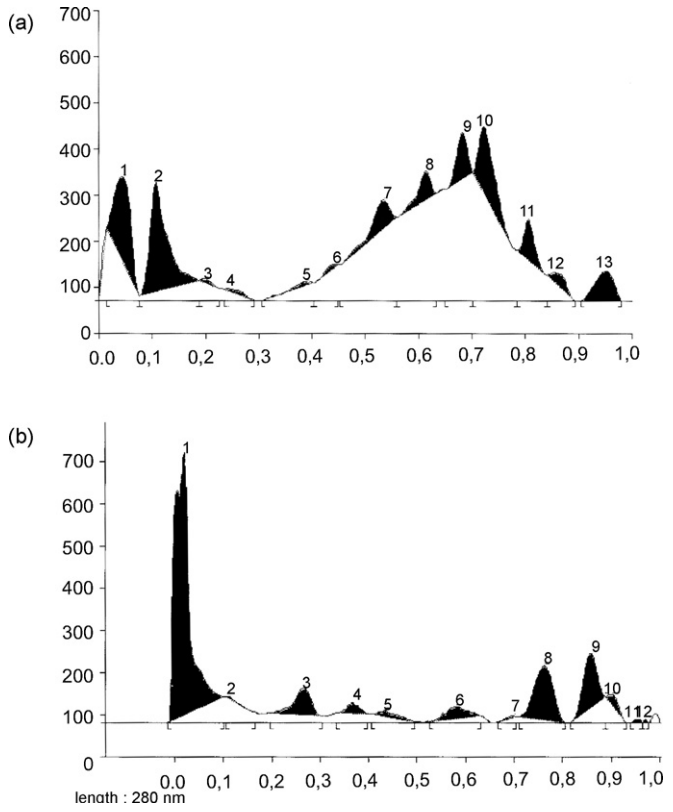

Fig. 1. HPTLC chromatograms of bark (a) and leaf (b). Bark and leaf of *Holoptelea integrifolia* were subjected to HPTLC for fingerprinting. Bark (a) and leaf (b) gave13 and 12 spots, respectively, in the solvent system of hexane:ethyleacetate (2:1) for leaf, chloroform:methanol:water (5:2:0.5).

3.2. Antioxidant activity

3.2.1. Total phenolic content

Total phenolic content was expressed as GAE (gallic acid equivalents) in which, MSBE has shown greater amount of GAE than the MLE. Total phenolic contents of MLE and MSBE were 57.71 ± 1.45 and 78.53 ± 1.26 mg/g weight of dry extract, respectively (values are mean ± S.D. of three individual experiments).

3.2.2. Antioxidant activity (DPPH free radical scavenging)

Peak areas of control and test samples were compared for the determination of percentage inhibition. MLE and MSBE stock solutions at the concentration of 3 mg/ml have shown 69.0 ± 0.36 and 81.41 ± 1.15% inhibition, respectively (Fig. 2) and the IC_{50} values obtained were 50.36 ± 0.59 and 37.66 ± 0.48 μg/well and ascorbic acid was 10 ± 0.66 μg/ml (values are mean ± S.D. of three individual experiments).

3.3. Anti microbial activity

3.3.1. Sensitivity test

The methanolic extracts from the leaves and stem bark of *Holoptelea integrifolia* has shown inhibition effects on the growth of all the organisms tested, but their efficiency in inhibition was varied from one organism to another. In almost all,

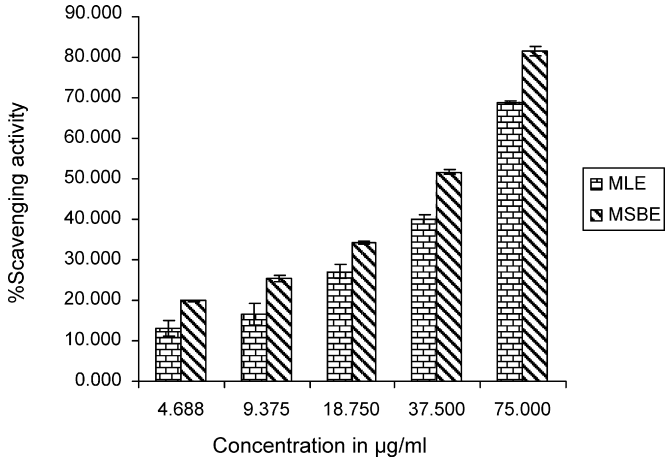

Fig. 2. Different concentrations of MLE and MSBE and their corresponding antioxidant activity evaluated by DPPH free radical scavenging ability. Methanolic extracts of leaves (MLE) and stem bark (MSBE) of *Holoptelea* were serially diluted, ranging from 75 μg to 4.6 μg/ml, and subjected to DPPH free radical scavenging activity in which, MSBE has shown greater antioxidant activity than MLE at any given concentration. The IC_{50} values obtained for MLE and MSBE are 50.36 ± 0.59 and 37.66 ± 0.48 μg/well. Well final volume is 250 μl (values are mean ± S.D. of three individual experiments).

the tested organisms' growth was inhibited by both MLE and MSBE. MSBE has shown higher range of inhibition diameter (IDZ) from 11.3 to 20.4 mm, whereas MLE has shown inhibition range of 9.6–14.9 mm. *Candida tropicana* is more sensitive and *Bacillus cerculences* is least sensitive to MSBE. *Sacharomyces cerviceae* and *Candida tropicana* have shown higher and equal sensitivity to MLE. Chloramphenicol and Amphotericin B have shown IZD ranged from 18 ± 0.12 to 20.4 ± 1.02 mm at a concentration of 30 μg/bore. All IZD corresponding to test organisms are tabulated in Table 1.

3.3.2. MIC and MMC determination

The methanolic extract of leaves and stem bark of *Holoptelea integrifolia* were tested at different concentrations for antimicrobial activity, the extent of their inhibitory activities against the test organisms could be well understood only by comparing the MICs and MMCs values obtained. MICs and MMCs of different microbes are given in Table 1. The results indicated that *Candida tropicana* (MIC: 39 μg/ml) is most sensitive to MSBE and *Sacharomyces cerviceae* (MIC: 156.2 μg/ml) and *Candida tropicana* (MIC: 156.2 μg/ml) are most and equal sensitive organisms to MLE. Their MICs are raged from 156.2 to 1250 μg/ml and 39 to 625 μg/ml for MLE and MSBE, respectively. The lowest MMC observed was 39.0 μg/ml for *Candida tropicana* against MSBE. MIC and MMC values for Chloramphenicol and Amphotericin B were ranged form 4.8 to 19.50 μg/ml.

3.4. Wound-healing activity

3.4.1. Excision model

The area of wound was measurement on the days 1, 4, 7, 10, 13, 16, 19 and 21 days of post surgery in all the groups. The control group, treated with simple ointment, has shown little contraction compared with MLE and MSBE compounds. A very rapid closure of the wound in the both MLE and MSBE treated groups observed between 4 and 8 days of post surgery ($p < 0.01$). After day 8 of post surgery, wound closure was gradual till the total closure of the wound. However, in the standard group, treated with Nitrofurazone, has shown gradual closure of the wound (Fig. 3) and the percentage of wound healing of the extracts against the post surgery days are presented in Fig. 4.

On the day 16 of post surgery, mean wound area of MLE and MSBE and standard groups were 13.42 ± 1.79, 9.72 ± 2.16 and 23.07 ± 9.96 mm^2, respectively, but in control group wound area was 142.23 ± 13.35 mm^2 (70%), indicating that both extracts are showing wound-healing property comparable to that of standard group. Total wound closure was observed by the 16 day of post surgery in all the treated groups and by 21 day in control group.

3.4.2. Incision model

3.4.2.1. Tensile strength. On the day 10 post surgery, breaking strength of the skin was measured using the Universal testing

Table 1
Antimicrobial activity of MLE and MSBE on different microbes and their corresponding IZD, MIC and MMCs

S. No.	Microbe	IZD (mm^2) 1.5 mg/bore			MIC (μg/ml)			MMC (μg/ml)		
		MLE	MSBE	Standard	MLE	MSBE	Standard	MLE	MSBE	Standard
1	*Bacillus cerculences*	12.6 ± 2.3	11.3 ± 0.6	18.7 ± 3.11	312.5	625	6.25	156.2	156.2	3.13
2	*Pseudomonas aeruginosa*	13.2 ± 1.6	15.2 ± 1.8	20.8 ± 2.3	312.5	625	3.13	156.2	312.5	3.13
3	*Bacillus subtilis*	9.9 ± 1.7	14.6 ± 2.5	21.2 ± 1.65	625	312.5	25.0	312.5	156.2	12.5
4	*Klebsiella aeruginosa*	12.6 ± 2.6	16.5 ± 2.3	19.8 ± 1.19	312.5	78.13	25.0	312.5	78.13	12.5
5	*Staphylococcus aureus*	9.6 ± 2.1	13.4 ± 1.5	20.6 ± 2.22	1250	625	12.5	1250	625	6.25
6	*Escherichia coli*	11.2 ± 2.0	11.6 ± 2.3	22.8 ± 4.1	312.5	312.5	3.12	156.2	156.2	3.12
7	*Aspergillus niger*	14.9 ± 1.7	12.8 ± 1.7	23.1 ± 3.22	625	312.5	12.5	312.5	156.2	6.25
8	*Sacharomyces cerviceae*	12.6 ± 0.9	16.2 ± 1.4	17.5 ± 2.26	156.2	78.5	6.25	156.2	78.2	3.13
9	*Candida krusei*	10.9 ± 1.6	17.9 ± 2.2	21.6 ± 2.15	625	78.1	62.5	312.5	39	3.13
10	*Candida albicans*	12.1 ± 1.1	18.6 ± 1.2	20.3 ± 3.21	312.5	78.1	6.25	312.5	78.1	6.25
11	*Candida tropicana*	14.5 ± 1.0	20.4 ± 0.9	20.2 ± 2.32	156.2	39.0	3.13	156.2	39	1.56

Holoptelea integrifolia leaves and stem bark extracts were screened for antimicrobial activity. All the test organisms are more sensitive to MSBE than the MLE except *Bacillus cerculences* and *Pseudomonas aeruginosa*, which are more sensitive to MLE. *Escherichia coli* is equally sensitive to both the extracts. Chloramphenicol and Amphotericin B are the standards for bacteria and fungus, respectively (values are mean ± S.D. of three replicates).

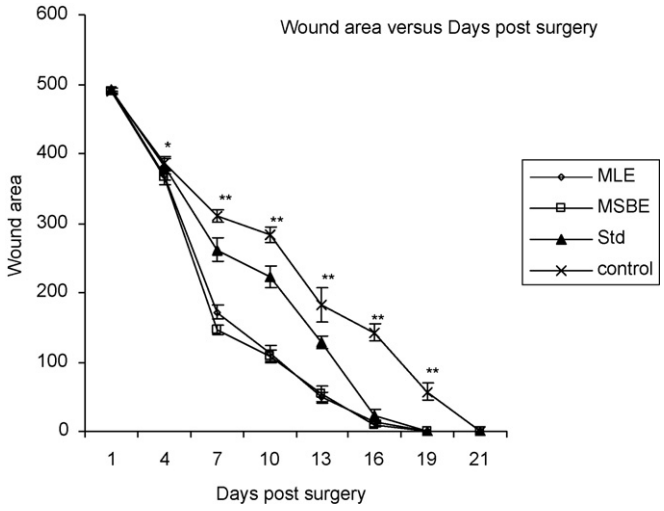

Fig. 3. Comparative mean wound area of different groups at different days after surgery ($n=5$) (*$p<0.05$, **$p<0.01$). Both the extracts MLE and MSBE are accelerating the wound-healing process. Both the extracts, MLE and MSBE have shown 100% healing on day 16 of post surgery but in standard group and control group complete healing appeared on days 19 and 21 of post surgery, respectively (values are mean ± S.D. of six rats).

machine (Shimadzu, Switzerland) (Saha et al., 1997). The breaking strengths and tensile strengths of the each group presented in Table 2, in which both the MLE and MSBE groups breaking strengths were 721 ± 22.1 and 988 ± 13.7 g, respectively. Both MLE and MSBE extracts have shown high breaking strength than that of control group. MSBE has shown higher breaking strength and higher collagen content ($p<0.05$) than the other groups.

3.4.3. Hydroxyproline estimation

Hydroxyproline is not directly coded by DNA, however, proline is hydroxylated to form hydroxyproline after protein synthesis. Hydroxyproline is a major component of the protein collagen. Hydroxyproline and proline play key roles for collagen stability. They permit the sharp twisting of the collagen helix.

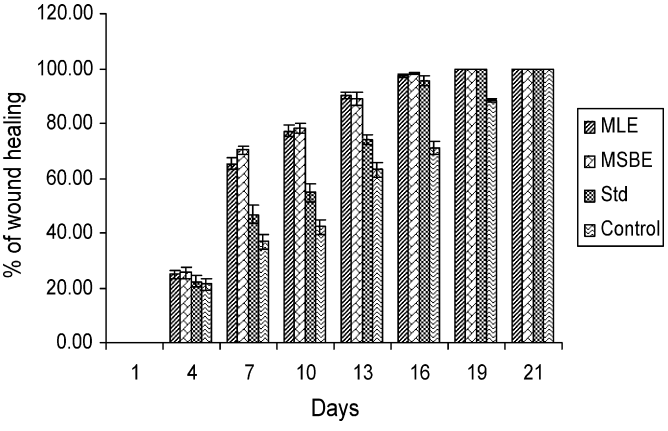

Fig. 4. Percentage of wound healing after surgery in different groups. MLE and MSBE are showing more percent of healing than the control group. Both the extracts are showing more healing activity during the days 3–7 than the other days. Complete healing was recorded on day of 16 for MLE and MSBE but in control group, it has taken 21 days to heal.

Table 2
Breaking (g) and tensile strengths (g/mm^2) of different groups

S. No.	Group	Breaking strength	Tensile strength	Hydroxyproline (mg/g tissue)
1	Control	521 ± 21.3	2.61	51.2 ± 4.21
2	Standard	756 ± 15.2	3.78	66.11 ± 2.53
3	MLE	721 ± 22.1	3.60	65.33 ± 3.11
4	MSBE	988 ± 13.7	4.94	72.22 ± 4.11

This table shows breaking, tensile strengths and collagen content of different test compound treated groups. A 200 mm^2 skin strip was isolated from the incision wound area and subjected to universal testing machine to evaluate breaking strength. MSBE group has shown more tensile strength (988 g) and collagen content than the other groups (values are mean ± S.D. of six rats).

They help on providing stability to the triple-helical structure of collagen by forming hydrogen bonds. Hydroxyproline is found in few proteins other than collagen. The only other mammalian protein which includes hydroxyproline is elastin. For this reason, hydroxyproline content has been used as an indicator to determine collagen content (Nelson and Cox, 2005). Hydroxyproline contents were found to be increased significantly in the groups treated with MLE and MSBE (65.33 ± 3.11 and 72.22 ± 2.11, respectively) than the control group (51.2 ± 4.21) (Table 2), which implies more collagen deposition in treated groups than the control group.

3.5. Histopathological observations

Treatment of rat wounds with MLE and MSBE ointments have led to reduce scar formation and enhanced fibroblast proliferation, angiogenesis, keratinazation and epithelisation as compared to vehicle treated group or control group. Photograph of skins are presented in Fig. 5.

4. Discussion

When a wound occurs and is exposed to external environment, it is more prone to attack by microbes, which invade through the skin and delay the natural wound-healing process. Reactive oxygen species (ROS, includes oxygen-derived radical as well as non-radical oxidants), often loosely termed "oxidants," are vital part of healing and serve as cellular messengers that drive numerous aspects of molecular and cell biology. ROS can trigger the various beneficial pathways of wound healing, for example, at micromolar concentration concentrations of hydrogen peroxide can promote vascular endothelial growth factor (VEGF) expression in keratinocytes (Khanna et al., 2001). In the inflammation phase of healing neutrophils and macrophages are attracted into the injured tissue by various chemotactic factors. They locate, identify, phagocytize, kill, and digest microorganisms and eliminate wound debris through their characteristic "respiratory burst" activity and phagocytosis (Clark and Moon, 1999). Superoxide is rapidly converted to membrane permeable form, H_2O_2, by superoxide dismutase activity. Release of H_2O_2 may promote formation of other oxidants that are more stable (longer half-life) including, hypochlorous acid, chloramines, and aldehydes. Taken together, this suggests that the wound site

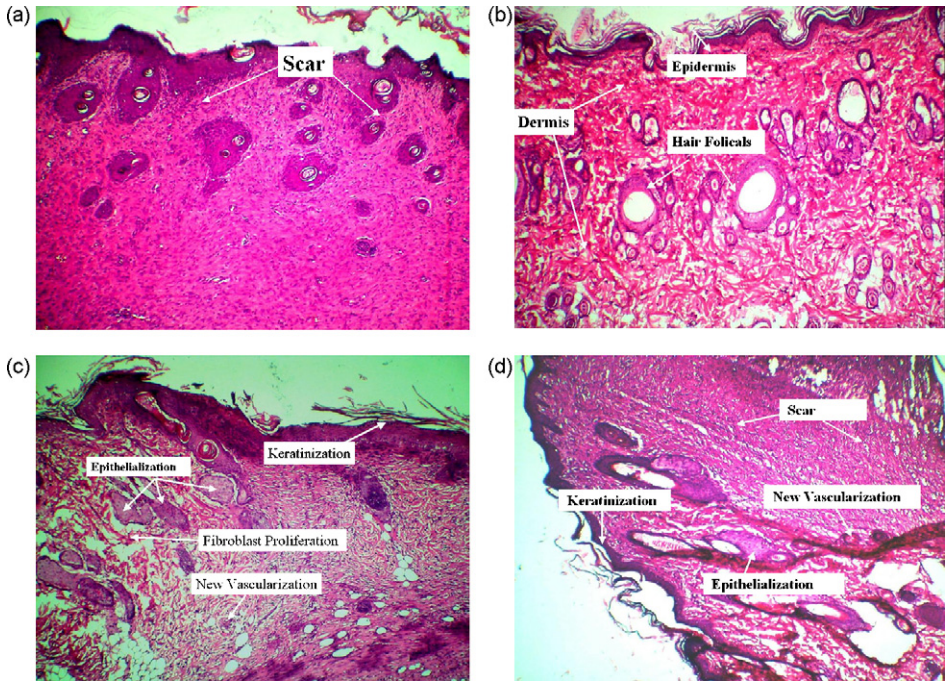

Fig. 5. Histopathology of skin in control and treated group. Skin histopathology of control group (a), normal skin (b), MLE treated group (c) and MSBE treated group (d). Photographs are showing clear evidence for epithelisation, neovascularization, keratinization and low scar area formation in treated groups than the control group.

is rich in oxidants along with their derivatives such as chloramines (Chandan et al., 2002). At high concentrations, ROS can induce severe tissue damage and even lead to neoplastic transformation. Which further impede the healing process by causing damage to cellular membranes, DNA, proteins and lipids as well (Martin, 1996) Further, elevated lipid peroxide levels have also been demonstrated in certain inflammatory skin lesions such as traumatic wounds and radiation dermatitis (Niwa et al., 1987) Therefore, cells have developed mechanisms to detoxify ROS. In general, there are two major strategies which convey partial resistance against oxidative stress to most cell types: small antioxidant molecules like ascorbate, polyunsaturated fatty acids or sugars (mainly mannitol), and ROS-scavenging enzymes, such as superoxide dismutases (SOD), catalase, and various peroxidases (Heike Steiling et al., 1999). Hence, if a compound having antioxidant potentials and antimicrobial activity additionally, it can be a good therapeutic agent for accelerating the wound-healing process.

Results obtained in this study conform the wound-healing activity of *Holoptelea integrifolia*, which also possesses antimicrobial and antioxidant activity. Antioxidant activity was assessed by DPPH scavenging method where MSBE was found to be most potent antioxidant than the MLE, for which IC_{50} values are 37.66 ± 0.48 and 50.36 ± 0.59 μg/well, respectively. The higher phenolic content of MSBE (TPC: 78.53 ± 1.26 mg/g) might have contributed to higher antioxidant activity of MSBE than MLE (TPC: 57.71 ± 1.45 mg/g) (Maisuthisakul et al., 2005). In the preliminary screening of antimicrobial activity, using agar diffusion method, MSBE has shown higher zone of inhibition than MLE for which IZD ranged from 11.3 to 20.4 mm. The same is reproduced in the MIC where the lowest MIC obtained for MSBE is 39.0 μg/ml and for MLE is 156.2 mm, MIC and MMC were varied from one organism to another and all the microorganisms were more sensitive to MSBE compared to MLE. However, *Bacillus cerculences* and *Pseudomonas aeruginosa* microbes were found to be more susceptible to MLE than the MSBE. *Escherichia coli* was equally sensitive to both MLE and MSBE.

In the wound-healing studies, the wound closure time, wound contraction and breaking strengths were taken as parameters. In the both MSBE and MLE treated groups wound closure time was 16 days, where as in control group and standard group they are 21and 17 days, respectively. As shown in Fig. 3, around 98% of wound healing was recorded in both the treated groups but only 70.23% was recorded in control group on 16 day of post surgery. Collagen formation and concentration both will affect the breaking strength of the skin (Udupa, 1994). As shown in Table 2, breaking strength is more in MSBE group than the other group, which implies that MSBE and MLE causes deposition of collagen at the site of wound healing.

5. Conclusion

In conclusion, this study conforms the promising wound-healing activity of *Holoptelea integrifolia*. Results obtained in the present study have shown that both the extracts, MLE and MSBE, are active against the growth of the microbes such as *Bacillus subtilis*, *Bacillus cerculences*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella aeruginosa*, *Candida albicans*, *Saccharomyces serviseae*, *Aspergillus niger*, *Candida tropicana* and *Candida krusei*. Both extracts possess anti-oxidant activity. Hence, the external application of

these extracts on the wound prevented the microbes to invade through the wound, resulting protection of wound against the infections of the various microorganisms. At the same time, external application of the extracts entrapped the free radicals liberated from the wound surrounding cells, which are having inherent machinery to protect the cells form the microbes. Hence, the synergistic effect of both antimicrobial and antioxidant activity accelerated the wound-healing process.

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